IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Gerald P. Murphy *et al.*	Confirmation No. 7366
Application No.: 09/016,737	Examiner: M. Davis
Filed: January 30, 1998	Technology Center/Art Unit: 1642
For: ISOLATION AND/OR PRESERVATION OF DENDRITIC CELLS FOR PROSTATE CANCER IMMUNOTHERAPY	DECLARATION UNDER 37 C.F.R. § 1.131
Customer No.: 20350	

Commissioner for Patents
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I, Alton Boynton, state as follows:

1. I am an inventor of the invention claimed in U.S. Patent Application No. 09/016,737, filed January 30, 1998 (herein "the '737 application"). The '737 application is a U.S. national phase application under 35 U.S.C. § 371 of Application No. PCT/US96/12389, filed July 31, 1996, which is a continuation-in-part of U.S. Patent Application No. 08/509,254, filed July 31, 1995, issued as U.S. Patent No. 5,788,963 (herein "the '254 application").

2. The invention disclosed and claimed in the '737 application and the '254 application (herein "the invention") is directed to, *inter alia*, a composition comprising an isolated cell population having human dendritic cells, wherein the cell population has been cultured in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 4 (IL-4). The cultured cell population is exposed *in vitro* to a soluble prostate antigen and the exposed cell population has an increased ability to activate T cells specific to the prostate antigen as compared to a similar isolated cell population cultured in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin 4 (IL-4) that has not been exposed *in vitro* to the prostate antigen. The composition of cultured cells comprising human dendritic cells can be

exposed to a prostate antigen that includes a lysate of LNCaP cells, a membrane preparation of LNCaP cells, a lysate of prostate tumor cells from a prostate cancer patient, a membrane preparation of prostate tumor cells from a prostate cancer patient, isolated prostate specific membrane antigen (PSMA), purified prostate specific membrane antigen (PSMA), various peptides from a prostate antigen, purified prostate specific antigen (PSA), or a purified prostate mucin antigen recognized by monoclonal antibody PD41. The composition can comprise extended life dendritic cells or cells that have been cryopreserved.

3. I understand that pending claims 23, 24, and 31-36 of the '737 application currently remain rejected under 35 U.S.C. § 103(a) as being obvious over US Patent 5,643,786 to Cohen *et al.*, filed January 27, 1995, in view of Sallusto *et al.*, *J. Exp. Med.* 179:1109-1118, 1994 and Inaba *et al.*, *J. Ex. Med.* 166:182-194, 1987. Further, I understand that claim 26 remains rejected under 35 U.S.C. § 103 as being obvious over US Patent 5,643,786 to Cohen, in view of Sallusto *et al.*, and Inaba *et al.* (all cited above), and further in view of Lutz *et al. J. Immunol. Methods* 174:269-279, 1994. It is further understood that claims 28 and 29 remain rejected under 35 U.S.C. § 103 as being obvious over US Patent 5,643,786 to Cohen, in view of Sallusto *et al.*, and Inaba (all cited above) and further in view of Taylor *et al.*, *Cryobiol.* 27:269-278, 1990, and that claim 30 remains rejected under 35 U.S.C. § 103 as being obvious over US Patent 5, 643, 786 to Cohen, in view of Sallusto *et al.*, Inaba *et al.*, and further in view of Taylor *et al.* and Lutz *et al.* (all cited above).

4. Benjamin Tjoa, Gerald Murphy and I conceived of and reduced to practice the invention of the pending claims in the United States prior to January 27, 1995, or conceived of the claimed invention prior to January 27, 1995 coupled with due diligence in the United States from prior to January 27, 1995 to a subsequent reduction to practice or the filing of the parent application on July 31, 1995. As evidence of conception and reduction to practice of the claimed invention before January 27, 1995, or conception of the invention prior to January 27, 1995 coupled with due diligence from prior to January 27, 1995 to a subsequent reduction to practice or to the filing of the parent application on July 31, 1995, we have attached hereto Exhibit A.

5. Exhibit A is a copy of the manuscript entitled "*In vitro* propagated dendritic cells from prostate cancer patients as a component of prostate cancer immunotherapy", co-authored by

myself, Benjamin Tjoa, Sheila Erickson, Robert Barren III, Haakon Ragde, Gerald Kenny and Gerald Murphy (herein "the Tjoa *et al.* reference"). The Tjoa *et al.* reference published in The Prostate Volume 27:63-69, August 1995 and a copy is attached as Exhibit B. The manuscript was submitted to the editors of The Prostate on November 5, 1994 as indicated on the cover sheet for the manuscript and noted in the published paper on page 63, at the bottom of the right column. A revised version of the manuscript was resubmitted to the editors of The Prostate and accepted on April 2, 1995 which is subsequent to the effective filing date of US Patent 5, 643,786. The revised manuscript included the same experimental data as the original manuscript and did not substantively alter the scope of the subject matter disclosed.

6. Briefly, the Tjoa *et al.* reference describes studies reporting the *in vitro* propagation of dendritic cells from the peripheral blood of prostate cancer patients in GM-CSF and IL-4. After seven days in culture, the number of dendritic cells recovered were 20 to 50 fold higher than those isolated directly from peripheral blood and was described as similar to the number obtained in a comparable study with healthy individuals. The cultured dendritic cells were also shown to be capable of presenting tetanus toxoid to autologous T cells *in vitro*. Also, T cells for 2 of 4 patients were demonstrated to proliferate when cultured with the patients dendritic cells and a lysate from the human prostatic cell line LNCaP. The Tjoa *et al.* reference also describes additional prostate antigens under evaluation including prostate specific membrane antigen and tumor tissues from fresh prostatectomy specimens.

7. The studies disclosed in the Tjoa *et al.* reference are the same as those described in the '254 application at, for example pages 16-24, Figures 1 through 4, and throughout the specification. The same disclosure can be found at, for example, pages 19-27, Figures 1 through 4, and throughout the specification.

8. Sheila Erickson and Robert Barren III, at the time of publication of the Tjoa *et al.* reference, were technicians in the Cancer Research Division, Pacific Northwest Cancer Foundation, at Northwest Hospital working under the direction of either Benjamin Tjoa, Gerald Murphy, or myself.

9. Haakon Ragde and Gerald Kenny, at the time of publication of the Tjoa *et al.* reference, were physicians with Northwest Hospital who supplied patient blood samples and other tissue samples for ongoing research in our laboratories.

10. Accordingly, although Sheila Erickson, Robert Barren III, Haakon Ragde and Gerald Kenny are named as co-authors on the Tjoa *et al.* reference, as they did not participate in the conception of the subject matter disclosed and claimed in the '254 or the '737 applications, they are not inventors of the present invention.

11. Between November 5, 1994 and the filing date of the '254 application additional experiments were diligently carried out in the United States that describe, for example, the isolation of prostate specific membrane antigen (PSMA), T cell proliferative responses and cytotoxic responses induced by dendritic cells presenting PSMA, the use of cryopreserved dendritic cells to stimulate prostate specific T cells, cryopreservation of dendritic cells, and the use of PSMA peptides to stimulate T cells. The '254 application was also prepared, revised and filed during this time period.

12. In view of the above, a person of ordinary skill in the art would accept our possession of the claimed invention prior to January 27, 1995 or conception of the invention prior to January 27, 1995 coupled with due diligence from prior to January 27, 1995 to subsequent reduction to practice or to the filing of the parent application on July 31, 1995.

13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patent issuing from U.S. Patent Application No. 09/016,737.

Date: February 24, 2006

By: Alton Boynton
Alton Boynton

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IN VITRO PROPAGATED DENDRITIC CELLS FROM PROSTATE CANCER PATIENTS AS A COMPONENT OF PROSTATE CANCER IMMUNOTHERAPY[$]

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ABSTRACT

T cell mediated cancer immunotherapy requires efficient antigen presenting cells. Dendritic cells (DC's) are arguably the most efficient antigen presenting cells studied to date. Individuals with prostate cancer often undergo various therapies which may compromise their immune system, including the state of their DC precursors. We report the *in vitro* propagation of DC's from peripheral blood of patients with prostate cancer most of whom are in clinical stages D_1 or D_2 and have undergone radiation therapy. After 7 days in culture, the number of DC's recovered were 20-50 fold higher than those isolated directly from peripheral blood. This number is comparable to previous studies with healthy individuals. Cultured patients' DC's were capable of presenting tetanus toxoid to autologous T cells *in vitro*. Furthermore, T cells from 2/4 patients proliferated when cultured with their DC's and the lysate of a human prostate cancer cell line (LNCaP), demonstrating potential role of autologous DC's in prostate cancer immunotherapy studies.

INTRODUCTION

Evidence that the immune system recognizes the presence of neoplastic cells is supported by the existence of infiltrating lymphocytes in tumor tissues (1,2). However, despite the presence of these immune cells, the tumors often prevail and survive. Cancer immunotherapy seeks to manipulate the mechanics of the immune system such that cancer cells could not only be recognized but also eliminated. Advances in the understanding of T cell activation and recognition of target cells have begun to result in significant progress in the field of T cell-mediated cancer immunotherapy (3,4).

Recognition of the antigenic peptide bound to the major histocompatibility (MHC) proteins and interactions of co-stimulatory molecules and their ligands (e.g., B7/BB1 molecules on antigen presenting cells (APC's) to the CD28 molecule on the T cell surface) are required elements in T cell activation and proliferation (3,5). Among cells with antigen presenting capabilities, dendritic cells (DC) have been suggested to be the most efficient APC, providing all the necessary signals to activate naive and memory T cells (6). However, the low numbers of DC that can be isolated by various methods from any single tissue have been a giant hurdle in various aspects of DC studies, including analyses of their potential for use as APC's in cancer immunotherapy (6-13). In the recent years, several laboratories have devised different methods to amplify the number of DC by culturing DC precursors from different tissue origins (e.g., bone marrow, spleen, epidermis, and peripheral blood) in the presence of various mixtures of cytokines which include granulocyte/macrophage colony-stimulating factors (GM-CSF), tumor necrosis factor-α (TNF-α), and interleukin-4 (IL4) (14-21).

In order to evaluate the potential of DC's in prostate cancer immunotherapy, it is important to characterize DC's from patients, many of whom have undergone previous therapy that may have compromised their immune system. We have cultured DC's from

precursors in the peripheral blood of a group of prostate cancer patients using a combination of GM-CSF and IL4. A majority of these patients are in clinical stages D_1 or D_2 and have previously undergone radiation therapy. These cultured cells were characterized for the expression of lineage-specific determinants as well as other adhesion and co-stimulatory molecules to confirm their DC identity. These DC's were used as APC in T cell proliferation assays to demonstrate their capacity to present antigen to activate autologous T cells *in vitro*. This report describes in detail our observations and techniques.

MATERIALS AND METHODS

Cell lines and Reagents. LNCaP, a prostate cancer cell line (CRL 1740, ATCC, Rockville, MD), is maintained in culture in RPMI 1640, 2mM L-glutamine and 10% fetal calf serum (FCS; GIBCO-BRL, Gaithersburg, MD) (22). GM-CSF, recombinant human interleukin 2 (IL2) and interleukin 4 (IL4) were generous gifts from Amgen (Thousand Oaks, CA). Monoclonal antibodies Leu-6 (anti-CD1a), Leu-4 (anti-CD3), Leu-3a (anti-CD4), Leu-2a (anti-CD8), Leu-M3 (anti-CD14), anti-HLA-DR (MHC Class II), and BB1 (anti-B7/BB1) were purchased from Becton-Dickinson, San Jose, CA. Monoclonal antibodies SJ25-C1 (anti-CD19) and 3.9 (anti-CD11c) were purchased from Sigma, St. Louis, MO.

Prostate Cancer Patients. Patients with a histologic confirmation of prostatic cancer were selected for this study which included a signed informed consent. Fifty cc of heparinized peripheral blood for DC culture and other tests was drawn every 2 weeks during the period of observation which continues. Other non heparinized blood was drawn for prostate markers, including PSMA (23). Details regarding clinical stage, hematologic status, and other relevant treatments are recorded in table I. The American Urological System of staging was employed viz. B_2 = tumor confined to the prostate in both lobes, C_2 = large locally massive tumor, D_1 = positive pelvic lymph node, D_2 = metastatic disease.

DC Culture from Prostate Cancer Patients' Peripheral Blood Mononuclear Cells (PBMC). Peripheral blood was drawn from prostate cancer patients and was subjected to Lymphoprep (GIBCO-BRL, Gaithersburg, MD) density gradient centrifugation. The PBMC isolated were plated in 24 well plates (10^6-10^7 cells/well) and were incubated in a humidified incubator (37°C, 5% CO_2) for 90 minutes.

Non-adherent cells were removed with the supernatant and the wells are washed gently with warm (37°C) OPTIMEM medium (GIBCO-BRL, Gaithersburg, MD) and 5% FCS. Dendritic cell propagation medium (DCPM: OPTIMEM supplemented with 5% FCS, 500 units/ml GM-CSF and 500 units/ml IL-4) was added to the adherent cells (1 ml/well). These cells were cultured for 4-6 days before subcultured 1:3 in DCPM.

Characterization of Surface Antigen Expression of Cultured DC's. Cultured DC were incubated with monoclonal antibodies anti-CD1a,-CD3, -CD4, -CD8, -CD11c, -CD14, -CD19, -HLA-DR and -B7/BB1 for 30 minutes on ice, followed by a fluorescein-isothiocyanate labeled goat-anti-mouse Ig antibody for 30 minutes on ice. Fluorescence binding was analyzed using a FACS SCAN flow cytometer (Becton-Dickinson, San Jose, CA).

T Cell Proliferation Assays. One million prostate cancer patients' PBMC were plated in microtiter plates in T cell media (TCM) consisting of RPMI 1640, HEPES, 2-mercaptoethanol, L-glutamine and penicillin-streptomycin, supplemented with 10% human AB serum (Sigma, St. Louis, MO) and 1U/ml recombinant human IL 2. Ten thousand mitomycin-C inactivated autologous DC and antigens were added to the well prior to culture. The antigens used in these assays were tetanus toxoid at 500 ng/ml (TT; Sigma, St. Louis, MO) and the lysate of LNCaP cells from an equivalent of 10^5 LNCaP cells/ml. Lysate was prepared as described previously (24). Briefly, 10^7 LNCaP in 1 ml phosphate buffer saline (PBS) was subjected to cycles of repeated freezing in liquid nitrogen and quick thawing in a 37°C waterbath. The cell suspension was then added to the T cell proliferation assays.

RESULTS

1. Culture and Characterization of Prostate Cancer Patients' DC. A group of 10 prostate cancer patients were selected to be in this study. Their clinical profile is shown in table I. Most of these patients are in clinical stages D_1 or D_2, hormone refractory prostatic adenocarcinoma, and have undergone radiation therapy. Seven patients have undergone orchiectomy, among whom three have undergone Sr^{89} therapy (patients 2, 6, and 9). Table I shows that peripheral blood mononuclear cells (PBMC) yields from these three patients were considerably lower (1-3 x 10^5/cc) than those who were not given Sr^{89} therapy (10^6/cc).

Patients' DC were cultured from their PBMC as described in the materials and methods. After 4-7 days in culture, clusters of dividing cells started to form and became less adherent to the tissue culture flask. These cells increased in size and showed a typical dendritic morphology (figure 1). In addition to these slightly adherent cells, tightly adherent macrophages were also present. The average number of cells with dendritic morphology obtained after a 7 day culture is 2-7 x 10^6 from 50 ml peripheral blood, representing 4-14% of the starting number of PBMC cultured (table I).

In order to establish the identity of the cultured cells, they were harvested by pipetting (leaving tightly bound macrophages behind) and were subjected to flow cytometric analyses for surface expression of different protein markers for cells of hematopoeitic origin (figure 2). The cultured cells do not express lineage specific markers for T cells (CD3), B cells (CD19), or macrophage (CD14). CD1a, a marker for Langerhans cells, is expressed at a high level early in the culture, but the level decreases when the cells were maintained in cultured for more than 14 days. CD11c (beta-2-integrin), B7/BB1 and HLA-DR are also expressed by these cells, confirming further the identity of the cultured DC's. These cultures ceased to expand after 2 passages, although

the DC's maintain their characteristic morphology and surface antigen expression for up to one month when fed weekly with fresh dendritic cell propagation medium (DCPM).

2. Autologous T Cells from Prostate Cancer Patients' PBMC were Activated Against Tetanus Toxoid Presented by Cultured DC. In order to analyze the capacity of the cultured DC to present antigen to autologous T cells from the same patients, T cell proliferation assays were conducted as described in the materials and methods. Tetanus toxoid (TT) was chosen as a representative antigen in these experiments to show whether patients' memory T cells could be activated *in vitro*. Figure 3 shows that autologous T cells cultured with patients' DC's and TT proliferated at levels significantly higher than background levels (in the absence DC) as well as autologous mixed lymphocyte reaction (in the presence of DC without exogenous TT).

3. T Cells from Prostate Cancer Patients Could Be Activated Against Prostate Cancer Antigens Presented by Autologous DC's. The ability of patients' DC's to present TT to their own T cells evokes the question whether T cell responses specific for prostate cancer antigens could also be elicited *in vitro*. In this study, lysate of LNCaP (a metastatic prostate cancer cell line) cells was used as a representative prostate cancer antigen (22). Figure 4 shows that significant increases in ^{3}HTdR incorporation were observed in 2/4 cases when both DC's and LNCaP lysates were included in the assays.

4. T Cells Proliferated Include Both Helper and Cytolytic Populations. T cells proliferated against LNCaP lysate were expanded for 2 weeks and were subjected to flow cytometric analysis to determine the representation of the two T cell subtypes: cytolytic T lymphocytes (CTL) and helper T cells (T_H). Our analyses showed

that CTL's (CD8+) represent 40-50% of the T cells activated against the lysate of LNCaP cells (figure 5).

DISCUSSION

Various methods of culturing human DC's from different precursors (e.g., PBMC, bone marrow, and $CD34^+$ cells) have been reported (17-19,21). One of the major concern in prostate cancer immunotherapy studies is the state of cancer patients' immune system components that may have been compromised during the previous cancer therapies. Our study, involving a group individuals with advance stages of prostate cancer (table I), shows that functional DC's can be isolated and propagated *in vitro*. The DC yield from these patients ranges 2-7 x 10^6 cells from 50 ml peripheral blood or 4-14 % of the starting number of PBMC cultured. This figure is a significant improvement (20-50 fold) from the dendritic cell yield purified directly from PBMC (0.1-1%) and is comparable with those isolated from peripheral blood of normal individuals (9-11,19).

Surface antigen expression (lack of expression of lineage-specific antigens: CD3, CD14, and CD19 and high expression of CD1a, HLA-DR, and B7/BB1) are in agreement with previous reports on DC's cultured using various methods (14-21). Furthermore, the expression of CD11c indicates that these DC's are functionally mature (25,26).

DC's propagated with this methods stop dividing after 2 passages. Efforts to generate long-term DC lines have been reported in the mouse system by Paglia et. al. (27) This group used a recombinant retrovirus carrying the env^{AKR}-myc^{MH2} fusion gene to immortalize splenic DC (27). Attempts to generate long-term human DC lines are also being pursued in our lab in our continuing search for a way to generate a large number of APC for cancer immunotherapy studies.

In the present study, antigens were introduced to DC's at the start of the T cell proliferation assay. Other methods of introduction of antigen to DC cytosol to be processed and presented on the cell surface (e.g., osmotic lysis of pinocytic vesicles and the use of pH-sensitive liposomes) have been previously reported (28-31). These methods are currently being tested for effectiveness in our lab.

The present study shows *in vitro* T cell proliferation in 2/4 cases when LNCaP lysate was used as a representative prostate cancer antigen (figure 4) (23). We feel that the 2 negative experiments with LNCaP lysate reflect the limitations of using a crude cellular lysate with variable concentrations of prostate antigens. Other experiments underway in our laboratory using purified prostate-specific membrane antigen support this view. Several reports indicated that DC's elicit the proliferation of helper , cytolytic or both T cell populations (10, 18, 32-35). Our analyses showed that both populations are represented in the T cell populations activated against the LNCaP lysate (figure 5). The presence of both populations could be an important factor in launching an effective T cell response *in vivo*.

Several other prostate cancer specific antigens are currently being evaluated in our lab for their ability to be presented to patients' T cells. For example, the prostate specific-membrane antigen (PSMA) is one of the candidates for antigen in our immunotherapy study (23,36). This protein is recognized by the monoclonal antibody 7E11.C5 which has been used for *in vivo* imaging of metastatic prostatic carcinoma (37,38). We have also obtained tumor tissues from fresh radical prostatectomy specimens to be used as the antigen presented by autologous DC in *in vitro* T cell activation experiments as well as to be cultured for target cells in a cytotoxicity assay. Successes in *in vitro* studies will lead the way to a phase I clinical trial in patients with hormone refractory metastatic prostate cancer.

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Figure Legends

Figure 1. Morphology of cultured DC's isolated from the peripheral blood of a prostate cancer patient. Isolation and culture procedures were described in the materials and methods. The photo micrograph was taken at a 400X magnification.

Figure 2. Cell surface phenotype of peripheral blood-derived cells cultured for 7 days in the presence of GM-CSF and IL4. The isolation, culture and cell-labeling procedures were described in the materials and methods. The upper left histograms represents background fluorescence staining using the 2° antibody in the absence of the 1° antibody. Other histograms represent experiments with the indicated 1° antibodies. The populations were positive for CD1a, CD4, CD11c, B7/BB1, and HLA-DR and were negative for CD3, CD14, and CD19. These surface antigen expressions are characteristics of dendritic cells.

Figure 3. *In vitro* activation of patients' T cells against tetanus toxoid presented by autologous DC's. Assays were performed in triplicates in the presence and absence of tetanus toxoid (TT) as described in the materials and methods. The average ^{3}HTdR incorporated (cpm) is reported on the y axis as a measure of the extent of T cell proliferation. Individual standard deviations are shown. The x axis represents three different culture conditions as indicated. Increase in the ^{3}HTdR incorporated in wells in which DC's and TT were included compared to the controls indicate that presentation of TT by DC's are necessary for T cell proliferation.

Figure 4. In vitro activation of patient's T cells against LNCaP lysate presented by autologous DC's. PBMC from 4 different patients with prostate cancer were cultured in the presence and absence of autologous DC's and antigen (LNCaP lysate)

as described in the materials and methods. The average ^{3}HTdR incorporated (cpm) is reported on the y axis as a measure of the extent of T cell proliferation. Individual standard deviation is shown. The x axis represents three different culture conditions as indicated. Data from individual patient is shown by different bar graph patterns as indicated. T cell proliferation was observed in 2/4 cases (patients 1 and 4) when both DC's and LNCaP lysate were both present.

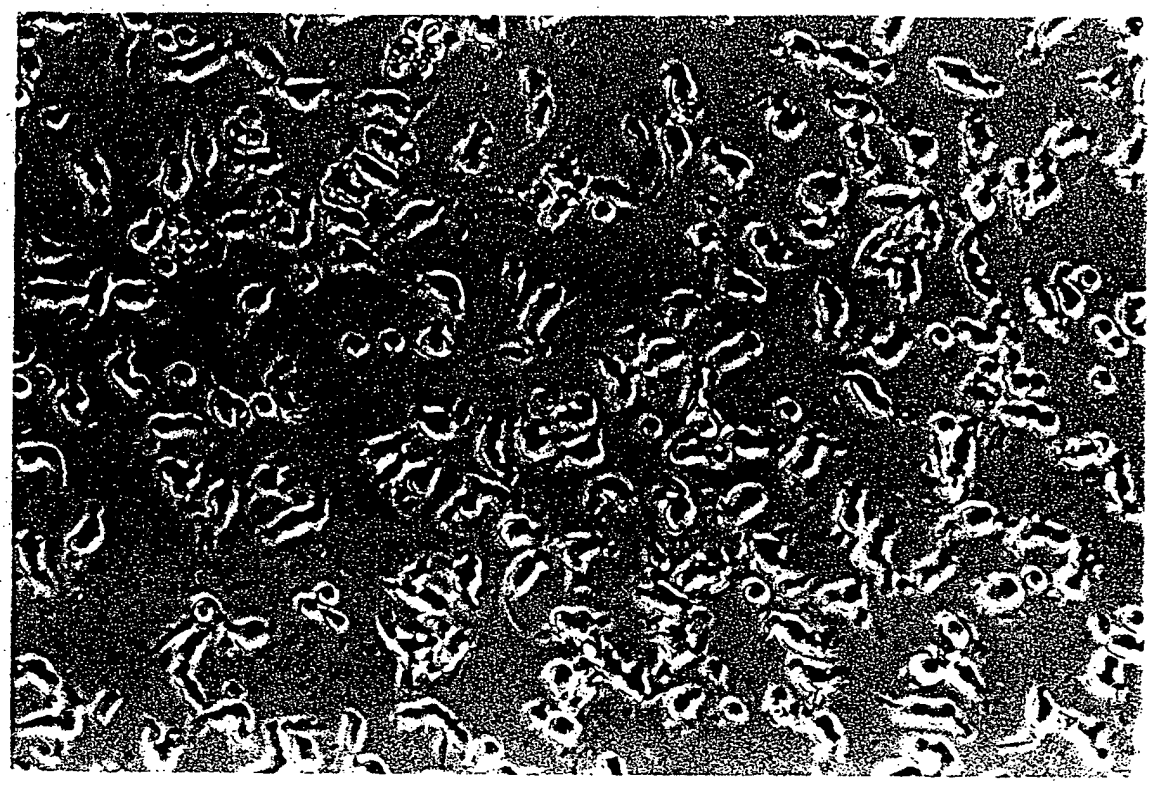
Figure 5. Population of T cells proliferated against LNCaP lysate. Patients' T cell cultures against LNCaP lysate were expanded in T cell propagation medium in the presence of mitomycin-C inactivated autologous DC and LNCaP lysate (equivalent to 10^4 LNCaP cells/ml). A 14 day old culture was harvested analyzed for the expression of CD8 by flow cytometry as described in the materials and methods. The background fluorescence represented by experiments with 2° antibody only is shown in dotted histogram and experiments using the anti-CD8 1° antibody are depicted by the solid histogram. $CD8^+$ cells account for 45% of the T cells proliferated against LNCaP lysate in this particular experiment.

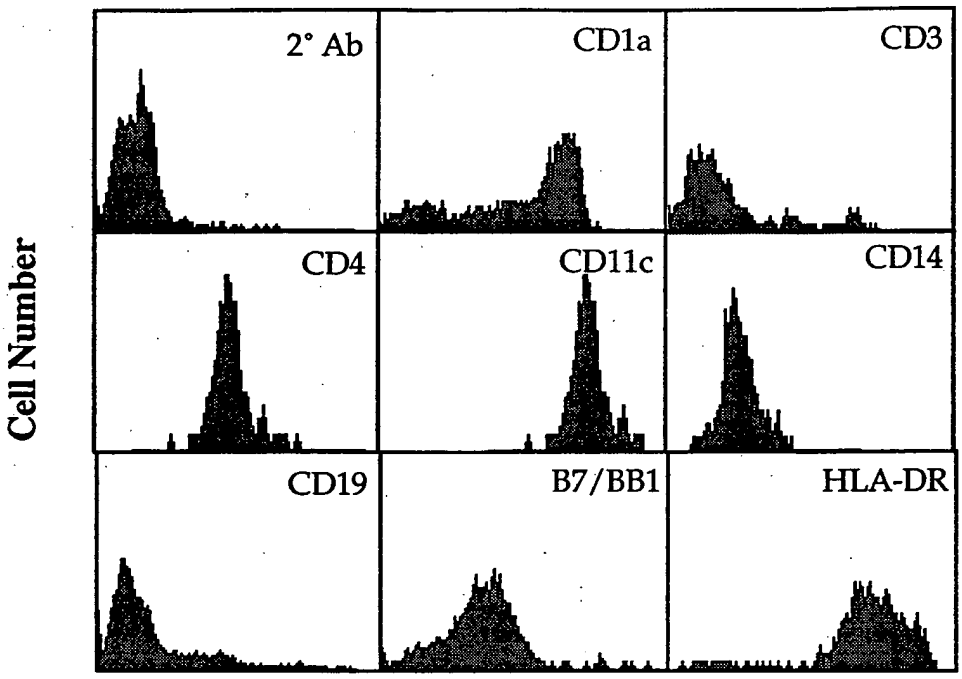
Table I. Clinical Profiles of Dendritic Cell Donors

Patient	Age	Clinical Stage	Hormonal Status	Bone Marrow Status	Hematocrit	Platelets < 100,000/cc	Duration on Program	Average PBMC yield# X 1000	DC yield*
1	64	D_1	Hormone Refractory, Leupron + Flutamide	Intact	45	0	7 months	1000	8-12%
2	71	D_2	Hormone Refractory, Post-Orchiectomy	Impaired, Ext. Radiation, 3 X Sr^{89}	34	+	8 months	100	4-10%
3	68	C_2	Hormone Refractory, Post-Orchiectomy	Ext. Radiation	46	0	5 months	1000	7-11%
4	65	D_2	Hormone Refractory, Post-Orchiectomy	Ext. Radiation	33	0	3 months	1000	10-14%
5	75	B_2	Non-Treated	Intact	47	0	2 months	1000	10-13%
6	70	D_2	Hormone Refractory, Post-Orchiectomy	Impaired Ext. Radiation, Multiple Sites, 1 x Sr^{89}	25	+	3 months	100	2-8%
7	80	D_2	Hormone Refractory, Post-Orchiectomy	Ext. Radiation, Multiple Sites	29	+	3 months	1000	5-10%
8	69	D_1	Hormone Refractory, Post-Orchiectomy, R_x Flutamide	Intact	45	0	8 months	1000	8-11%
9	62	D_2	Hormone Refractory, Post-Orchectomy, R_x Emcyt	Impaired, Ext. Radiation, Multiple Sites, 1 x Sr^{89}	33	0	10 months	300	6-9%
10	65	B_2	Intact	Intact	46	0	2 months	900	9-13%

#The number of PBMC isolated from 1 ml of blood

* The number of DC cultured for 7 days/the starting number of PBMC x 100%; The average volume of blood drawn every session = 50 ml

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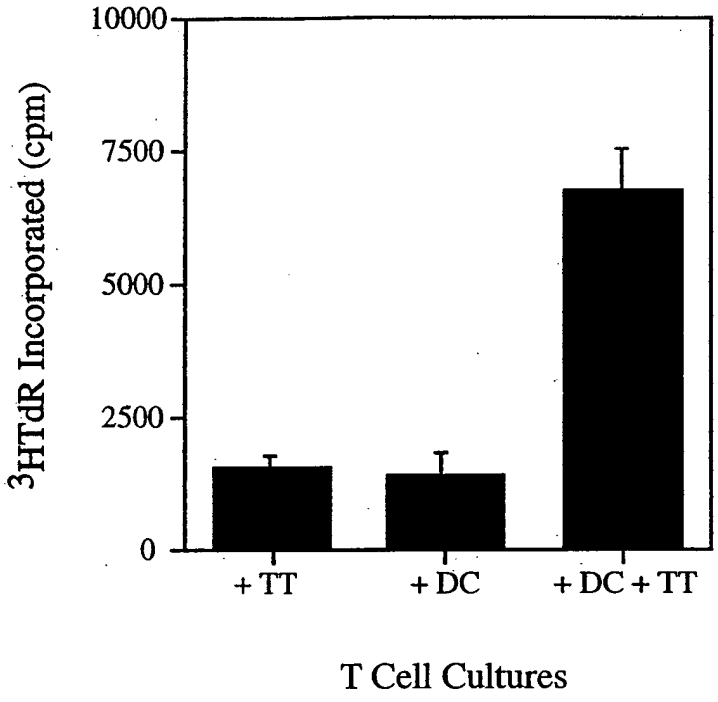
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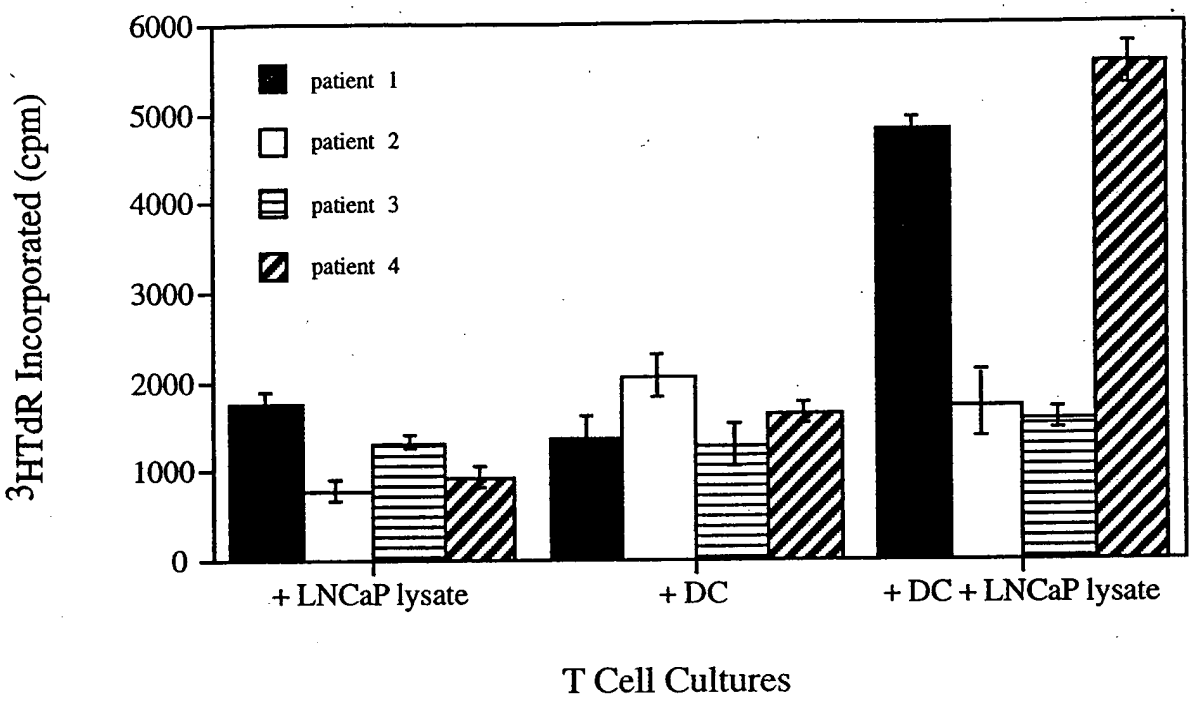
T Cell Cultures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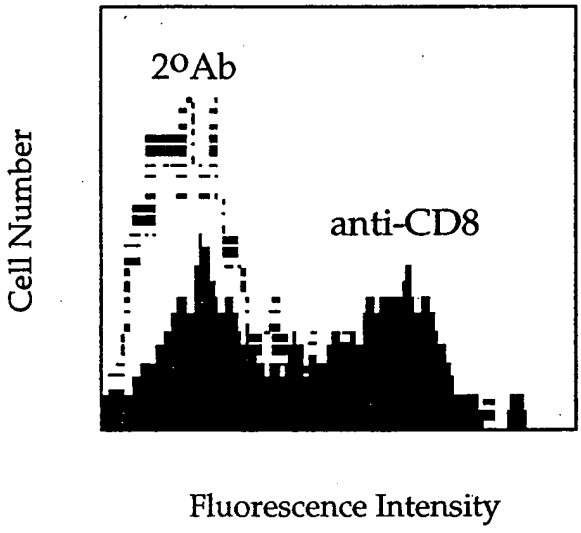
T Cell Cultures


2°Ab
anti-CD8
Cell Number
Fluorescence Intensity

The Prostate 27:63–69 (1995)

In Vitro Propagated Dendritic Cells From Prostate Cancer Patients as a Component of Prostate Cancer Immunotherapy

Benjamin Tjoa, Sheila Erickson, Robert Barren III, Haakon Ragde, Gerald Kenny, Alton Boynton, and Gerald Murphy

Pacific Northwest Cancer Foundation, Cancer Research Division (B.T., S.E., R.B., H.R., G.K., G.M.), and Department of Molecular Medicine (A.B.), Northwest Hospital, Seattle, Washington

ABSTRACT: T cell-mediated cancer immunotherapy requires efficient antigen-presenting cells. Dendritic cells (DCs) are arguably the most efficient antigen-presenting cells studied to date. Individuals with prostate cancer often undergo various therapies which may compromise their immune system, including the state of their DC precursors. We report the in vitro propagation of DCs from peripheral blood of patients with prostate cancer, most of whom are in clinical stages D_1 or D_2 and have undergone radiation therapy. After 7 days in culture, the number of DCs recovered were 20–50-fold higher than those isolated directly from peripheral blood. This number is comparable to findings of previous studies with healthy individuals. Cultured patients' DCs were capable of presenting tetanus toxoid to autologous T cells in vitro. Furthermore, T cells from 2 of 4 patients proliferated when cultured with their DCs and the lysate of a human prostate cancer cell line (LNCaP), demonstrating the potential role of autologous DCs in prostate cancer immunotherapy studies. © 1995 Wiley-Liss, Inc.

KEY WORDS: dendritic cells (DC), culture, T cell, immunotherapy, prostate cancer

INTRODUCTION

Evidence that the immune system recognizes the presence of neoplastic cells is supported by the existence of infiltrating lymphocytes in tumor tissues [1,2]. However, despite the presence of these immune cells, tumors often prevail and survive. Cancer immunotherapy seeks to manipulate the mechanics of the immune system such that cancer cells can not only be recognized but also eliminated. Advances in the understanding of T cell activation and recognition of target cells have begun to result in significant progress in the field of T cell-mediated cancer immunotherapy [3,4].

Recognition of the antigenic peptide bound to the major histocompatibility (MHC) proteins, and interactions of costimulatory molecules and their ligands (e.g., B7/BB1 molecule on antigen presenting cells (APCs) the CD28 molecule on the T cell surface), are required elements in T cell activation and proliferation [3,5]. Among cells with antigen-presenting capabilities, dendritic cells (DCs) have been suggested as the most efficient APCs, providing all the necessary signals to activate naïve and memory T cells [6]. However, the low numbers of DCs that can be isolated by various methods from any single tissue are a liability in various aspects of DC studies, including analyses of their potential for use as APCs in cancer immunotherapy [6–13]. In recent years, several laboratories have devised different methods to amplify the number of DCs by culturing DC precursors from different tissue origins (e.g., bone marrow, spleen, epidermis, and peripheral blood) in the presence of various mix-

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tures of cytokines which include granulocyte/macrophage colony-stimulating factors (GM-CSF), tumor necrosis factor-α (TNF-α), and interleukin-4 (IL4) [14–21].

In order to evaluate the potential of DCs in prostate cancer immunotherapy, it is important to characterize DCs from patients, many of whom have undergone previous therapy that may have compromised their immune systems. We cultured DCs from precursors in the peripheral blood of a group of prostate cancer patients, using a combination of GM-CSF and IL4. A majority of these patients are in clinical stages D_1 or D_2 and have previously undergone radiation therapy. These cultured cells were characterized for the expression of lineage-specific determinants, as well as other adhesion and costimulatory molecules, to confirm their DC identity. These DCs were used as APCs in T cell proliferation assays to demonstrate their capacity for presenting antigen to activate autologous T cells in vitro. This report describes in detail our observations and techniques.

MATERIALS AND METHODS

Cell Lines and Reagents

LNCaP, a prostate cancer cell line (CRL 1740, from the American Type Tissue Culture Collection, Rockville, MD), was maintained in culture in RPMI 1640, 2 mM L-glutamine, and 10% fetal calf serum (FCS; GIBCO-BRL, Gaithersburg, MD) [22]. GM-CSF, recombinant human interleukin-2 (IL2), and interleukin-4 (IL4) were generous gifts from Amgen (Thousand Oaks, CA). Monoclonal antibodies Leu-6 (anti-CD1a), Leu-4 (anti-CD3), Leu-3a (anti-CD4), Leu-2a (anti-CD8), Leu-M3 (anti-CD14), anti-HLA-DR (MHC class II), and BB1 (anti-B7/BB1) were purchased from Becton-Dickinson (San Jose, CA). Monoclonal antibodies SJ25-C1 (anti CD19) and 3.9 (anti-CD11c) were purchased from Sigma Chemical Co. (St. Louis, MO).

Prostate Cancer Patients

Patients with a histologic confirmation of prostatic cancer were selected for this study, which included a signed informed consent. Fifty ml of heparinized peripheral blood for DC culture and other tests were drawn every 2 weeks during the period of observation which continues. Other nonheparinized blood was drawn for prostate markers, including PSMA [23]. Details regarding clinical stage, hematologic status, and other relevant treatments are recorded in Table I. The American Urological System of staging was employed, i.e., B_2 = tumor confined to the prostate in both lobes, C_2 = large locally invasive tumor, D_1 = positive pelvic lymph node, and D_2 = metastatic disease.

DC Culture From Prostate Cancer Patients' Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood was drawn from prostate cancer patients and was subjected to Lymphoprep (GIBCO-BRL, Gaithersburg, MD) density gradient centrifugation. The isolated PBMCs were plated in 24-well plates (10^6–10^7 cells/well) and incubated in a humidified incubator (37°C, 5% CO_2) for 90 min. Nonadherent cells were removed with the supernatant, and the wells were washed gently with warm (37°C) OPTI-MEM medium (GIBCO-BRL, Gaithersburg, MD) and 5% FCS. Dendritic cell propagation medium (DCPM: OPTIMEM supplemented with 5% FCS, 500 units/ml GM-CSF, and 500 units/ml IL4) was added to the adherent cells (1 ml/well). These cells were cultured for 4–6 days before being subcultured 1:3 in DCPM.

Characterization of Surface Antigen Expression of Cultured DCs

Cultured DCs were incubated with monoclonal antibodies anti-CD1a, -CD3, -CD4, -CD8, -CD11c, -CD14, -CD19, -HLA-DR, and -B7/BB1 for 30 min on ice, followed by a fluorescein-isothiocyanate-labeled goat anti-mouse Ig antibody for 30 min on ice. Fluorescence binding was analyzed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

T Cell Proliferation Assays

One million prostate cancer patients' PBMCs were plated in microtiter plates in T cell media (TCM) consisting of RPMI 1640, HEPES, 2-mercaptoethanol, L-glutamine, and penicillin-streptomycin, supplemented with 10% human AB serum (Sigma) and 1 U/ml recombinant human IL2. Ten thousand mitomycin-C-inactivated autologous DCs and antigens were added to the well prior to culture. Antigens used in these assays were tetanus toxoid at 500 ng/ml (TT; Sigma) and the lysate of LNCaP cells from an equivalent of 10^5 LNCaP cells/ml. Lysate was prepared as described previously [24]. Briefly, 10^7 LNCaP in 1 ml phosphate-buffered saline (PBS) subjected to cycles of repeated freezing in liquid nitrogen and quick thawing in a 37°C waterbath. The cell suspension was then added to the T cell proliferation assays.

RESULTS

Culture and Characterization of Prostate Cancer Patients' DCs

A group of 10 prostate cancer patients was selected to be in this study. Their clinical profiles are shown in

TABLE I. Clinical Profiles of Dendritic Cell Donors

Patient	Age	Clinical stage	Hormonal status	Bone marrow status	Hematrocrit	Platelets <100,000/cc	Duration on program	Average PBMC yield × 1,000[a]	DC yield[b]
1	64	D_1	Hormone-refractory, leupron + flutamide	Intact	45	0	7 months	1,000	8–12%
2	71	D_2	Hormone-refractory, postorchiectomy	Impaired, ext. radiation, 3 × Sr^{89}	34	+	8 months	100	4–10%
3	68	C_2	Hormone-refractory, postorchiectomy	Ext. radiation	46	0	5 months	1,000	7–11%
4	65	D_2	Hormone-refractory, postorchiectomy	Ext. radiation	33	0	3 months	1,000	10–14%
5	75	B_2	Nontreated	Intact	47	0	2 months	1,000	10–13%
6	70	D_2	Hormone-refractory, postorchiectomy	Impaired, ext. radiation, multiple sites, 1 × Sr^{89}	25	+	3 months	100	2–8%
7	80	D_2	Hormone-refractory, postorchiectomy	Ext. radiation, multiple sites	29	+	3 months	1,000	5–10%
8	69	D_1	Hormone-refractory, postorchiectomy, R_x flutamide	Intact	45	0	8 months	1,000	8–11%
9	62	D_2	Hormone-refractory, postorchectomy, R_x cmcyt	Impaired, ext. radiation, multiple sites, 1 × Sr^{89}	33	0	10 months	300	6–9%
10	65	B_2	Intact	Intact	46	0	2 months	900	9–13%

[a]Number of PBMCs isolated from 1 ml of blood.
[b]Number of DCs cultured for 7 days/starting number of PBMCs × 100%; average volume of blood drawn every session = 50 ml.

Table I. Most of these patients were in clinical stages D_1 or D_2, hormone refractory prostatic adenocarcinoma, and had undergone radiation therapy. Seven patients had undergone orchiectomy, among whom 3 had undergone Sr^{89} therapy (patients 2, 6, and 9). Table I shows that peripheral blood mononuclear cells (PBMC) yields from these 3 patients were considerably lower (1–3 × 10^5/cc) than those who were not given Sr^{89} therapy (10^6/cc).

Patients' DCs were cultured from their PBMC as described in Materials and Methods. After 4–7 days in culture, clusters of dividing cells started to form and became less adherent to the tissue culture flask. These cells increased in size and showed a typical dendritic morphology (Fig. 1). In addition to these slightly adherent cells, tightly adherent macrophages were also present. The average number of cells with dendritic morphology obtained after a 7 day culture was 2–7 × 10^6 from 50 ml peripheral blood, representing 4–14% of the starting number of cultured PBMCs (Table I).

In order to establish the identity of the cultured cells, they were harvested by pipetting (leaving tightly-bound macrophages behind) and subjected to flow cytometric analyses for surface expression of different protein markers for cells of hematopoietic origin (Fig. 2). The cultured cells did not express lin-

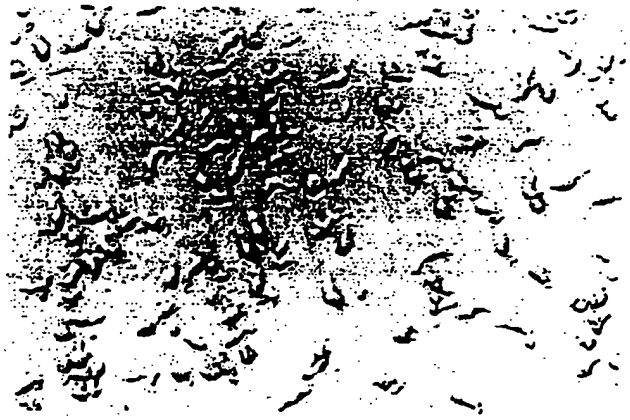

Fig. 1. Morphology of cultured DCs isolated from peripheral blood of a prostate cancer patient. Isolation and culture procedures were described in Materials and Methods. Photomicrograph, ×400.

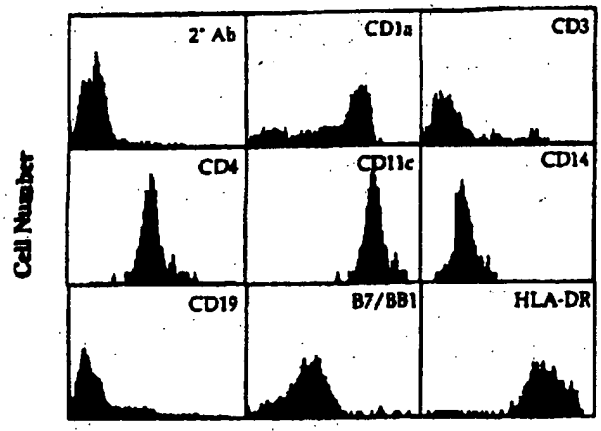

Fig. 2. Cell surface phenotype of peripheral blood-derived cells cultured for 7 days in presence of GM-CSF and IL-4. Isolation, culture, and cell-labeling procedures were described in Materials and Methods. Upper left histograms represent background fluorescence staining using the 2° antibody in the absence of the 1° antibody. Other histograms represent experiments with the indicated 1° antibodies. Populations were positive for CD1a, CD4, CD11c, B7/BB1, and HLA-DR, and were negative for CD3, CD14, and CD19. These surface antigen expressions are characteristic of dendritic cells.

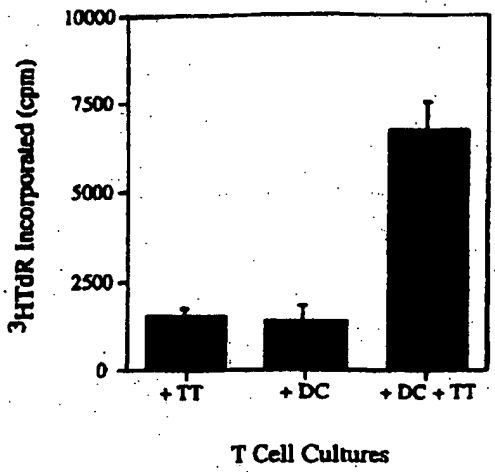

Fig. 3. In vitro activation of patients' T cells against tetanus toxoid presented by autologous DCs. Assays were performed in triplicate, in the presence and absence of tetanus toxoid (TT) as described in Materials and Methods. Average incorporated ^{3}HTdR (cpm) is reported on the y axis as a measure of the extent of T cell proliferation. Individual standard deviations are shown. The x axis represents three different culture conditions as indicated. Increase in the ^{3}HTdR incorporated in wells in which DCs and TT were included, compared to controls, indicates that presentation of TT by DCs is necessary for T cell proliferation.

eage-specific markers for T cells (CD3), B cells (CD19), or macrophage (CD14). CD1a, a marker for Langerhans cells, was expressed at a high level early in the culture, but the level decreased when the cells were maintained in cultured for more than 14 days. CD11c (beta-2-integrin), B7/BB1, and HLA-DR were also expressed by these cells, confirming further the identity of the cultured DCs. These cultures ceased to expand after two passages, although the DCs maintained their characteristic morphology and surface antigen expression for up to 1 month when fed weekly with fresh dendritic cell propagation medium (DCPM).

Autologous T Cells From Prostate Cancer Patients' PBMCs Were Activated Against Tetanus Toxoid Presented by Cultured DCs

In order to analyze the capacity of cultured DCs to present antigen to autologous T cells from the same patients, T cell proliferation assays were conducted as described in Materials and Methods. Tetanus toxoid (TT) was chosen as a representative antigen in these experiments to show whether patients' memory T cells could be activated in vitro. Figure 3 shows that autologous T cells cultured with patients' DCs and TT proliferated at levels significantly higher than control experiments, which include T cells cultured with TT only, as well as autologous mixed lymphocyte reaction (cultures in the presence of DCs without exogenous TT).

T Cells From Prostate Cancer Patients Could Be Activated Against Prostate Cancer Antigens Presented by Autologous DCs

The ability of patients' DCs to present TT to their own T cells evokes the question of whether T cell responses specific for prostate cancer antigens could also be elicited in vitro. In this study, lysate of LNCaP cells was used as a representative prostate cancer antigen [22]. Figure 4 shows that significant increases in ^{3}HTdR incorporation were observed in 2 of 4 cases when both DCs and LNCaP lysates were included in the assays.

Proliferating T Cells Include Both Helper and Cytolytic Populations

T cells proliferated against LNCaP lysate were expanded for 2 weeks and subjected to flow cytometric analysis to determine the representation of the two T cell subtypes: cytolytic T lymphocytes (CTL) and helper T cells (T_H). Our analyses showed that CTLs ($CD8^+$) represented 40–50% of the T cells activated against the lysate of LNCaP cells (Fig. 5).

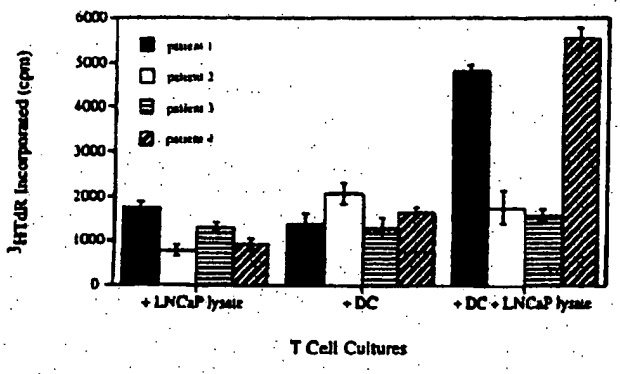

Fig. 4. In vitro activation of patients' T cells against LNCaP lysate presented by autologous DCs. PBMCs from 4 different patients with prostate cancer were cultured in the presence and absence of autologous DCs and antigen (LNCaP lysate), as described in Materials and Methods. The average incorporated ^{3}HTdR (cpm) is reported on the y axis as a measure of the extent of T cell proliferation. Individual standard deviations are shown. The x axis represents three different culture conditions as indicated. Data from individual patients are shown by different bar graph patterns as indicated. T cell proliferation was observed in 2 of 4 cases (patients 1 and 4), when both DCs and LNCaP lysate were present.

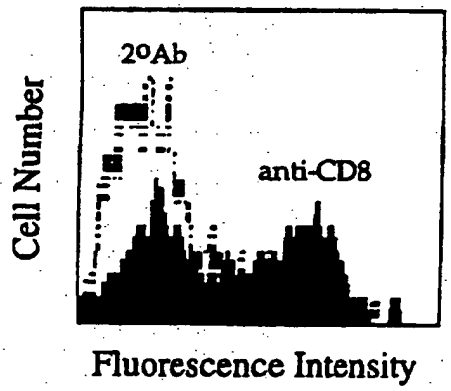

Fig. 5. Population of T cells proliferated against LNCaP lysate. Patients' T cell cultures against LNCaP lysate were expanded in T cell propagation medium in the presence of mitomycin-C-inactivated autologous DCs and LNCaP lysate (equivalent to 10^4 LNCaP cells/ml). A 14-day-old culture was harvested, and analyzed for the expression of CD8 by flow cytometry as described in Materials and Methods. Background fluorescence represented by experiments with 2° antibody only is shown in the dotted histogram, and experiments using the anti-CD8 1° antibody are depicted by the solid histogram. CD8$^+$ cells account for 45% of T cells proliferated against LNCaP lysate in this experiment.

DISCUSSION

Various methods of culturing human DCs from different precursors (e.g., PBMC, bone marrow, and CD34$^+$ cells) have been reported [17–19,21]. One major concern in prostate cancer immunotherapy studies is the state of cancer patients' immune system components that may have been compromised during previous cancer therapies. Our study, involving a group of individuals with advanced stages of prostate cancer (Table I), shows that functional DCs can be isolated and propagated in vitro. The DC yield from these patients ranges from 2–7 × 10^6 cells from 50 ml peripheral blood, or 4–14% of the starting number of PBMCs cultured. This figure is a significant improvement (20–50-fold) over the DC yield purified directly from PBMC (0.1–1%), and is comparable with those yields isolated from peripheral blood of normal individuals [9–10,19].

Surface antigen expression (lack of expression of lineage-specific antigens CD3, CD14, and CD19, and high expression of CD1a, HLA-DR, and B7/BB1) is in agreement with previous reports on DCs cultured by various methods [14–21]. Furthermore, the expression of CD11c indicates that these DCs are functionally mature [25,26].

DCs propagated with this method stop dividing after two passages. Efforts to generate long-term DC lines have been reported in the mouse system by Paglia et al. [27]. This group used a recombinant retrovirus carrying the env^{AKR}-myc^{MH2} fusion gene to immortalize splenic DC [27]. Attempts to generate long-term human DC lines are also being pursued in our lab, in our continuing search for a way to generate a large number of APCs for cancer immunotherapy studies.

In the present study, antigens were introduced to DCs at the start of the T cell proliferation assay. Using this technique, adequate antigen presentation was accomplished, as evidenced by the ability of TT and tumor cell lysate to elicit a cell proliferation response in autologous T cells. Other methods of introduction of antigen to DC cytosol to be processed and presented on the cell surface (e.g., osmotic lysis of pinocytic vesicles and the use of pH-sensitive liposomes) have been previously reported [28–31]. These methods are currently being tested for effectiveness in our laboratory.

The present study shows in vitro T cell proliferation in 2 of 4 cases when LNCaP lysate was used as a representative prostate cancer antigen (Fig. 4) [23]. We feel that the two negative experiments with LNCaP lysate reflect the limitations of using a crude cellular lysate with variable concentrations of prostate antigens. Other experiments underway in our laboratory using purified prostate-specific membrane antigen support this view. Several reports have indicated that DCs elicit the proliferation of helper, cytolytic, or both T cell populations [10,18,32–35]. Our analyses showed that both populations are rep-

resented in T cell populations activated against the LNCaP lysate (Fig. 5). The presence of both populations could be an important factor in launching an effective T cell response in vivo.

Several other prostate cancer-specific antigens are being evaluated in our laboratory for their ability to be presented to patients' T cells. For example, the prostate-specific membrane antigen (PSMA) is one of the candidates for antigen in our immunotherapy study [23,36]. This protein is recognized by the monoclonal antibody 7E11.C5, which has been used for in vivo imaging of metastatic prostatic carcinoma [37,38]. We have also obtained tumor tissues from fresh radical prostatectomy specimens to be used as the antigen presented by autologous DCs in vitro T cell activation experiments, as well as to be cultured for target cells in a cytotoxicity assay. Successes in in vitro studies will lead the way to a phase I clinical trial in patients with hormone-refractory metastatic prostate cancer. DCs may be used as the vehicle for presentation of tumor antigens in successful therapies for a number of additional cancers including: breast cancer, colon cancer, melanoma, and others in which tumor antigens have been recognized.

ACKNOWLEDGMENTS

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